Use of Proteomics and the Secretome and Exosporium of *Bacillus anthracis* in the Development of Bacterial Ghost-based Vaccines

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Proteome

The total proteins expressed by a genome at a particular moment in the life of a cell.

Proteomics is the study of proteomes.

Hundreds or thousands of proteins are studied simultaneously.

Standard proteomics investigations use 2D gel electrophoresis.

First dimension separation is achieved by isoelectric focusing. The proteins migrate in a pH gradient and when the point is reached where they have a net charge of zero (pI), they no longer migrate. Thus, separation is based on charge of the individual proteins.

Second dimension separation is at a 90 degree angle to the first. SDS polyacrylamide gel is used to separate proteins according to their size or molecular weight. The gel acts as a sieve with larger proteins migrate slower than smaller proteins.

Two Dimensional Gel Electrophoresis

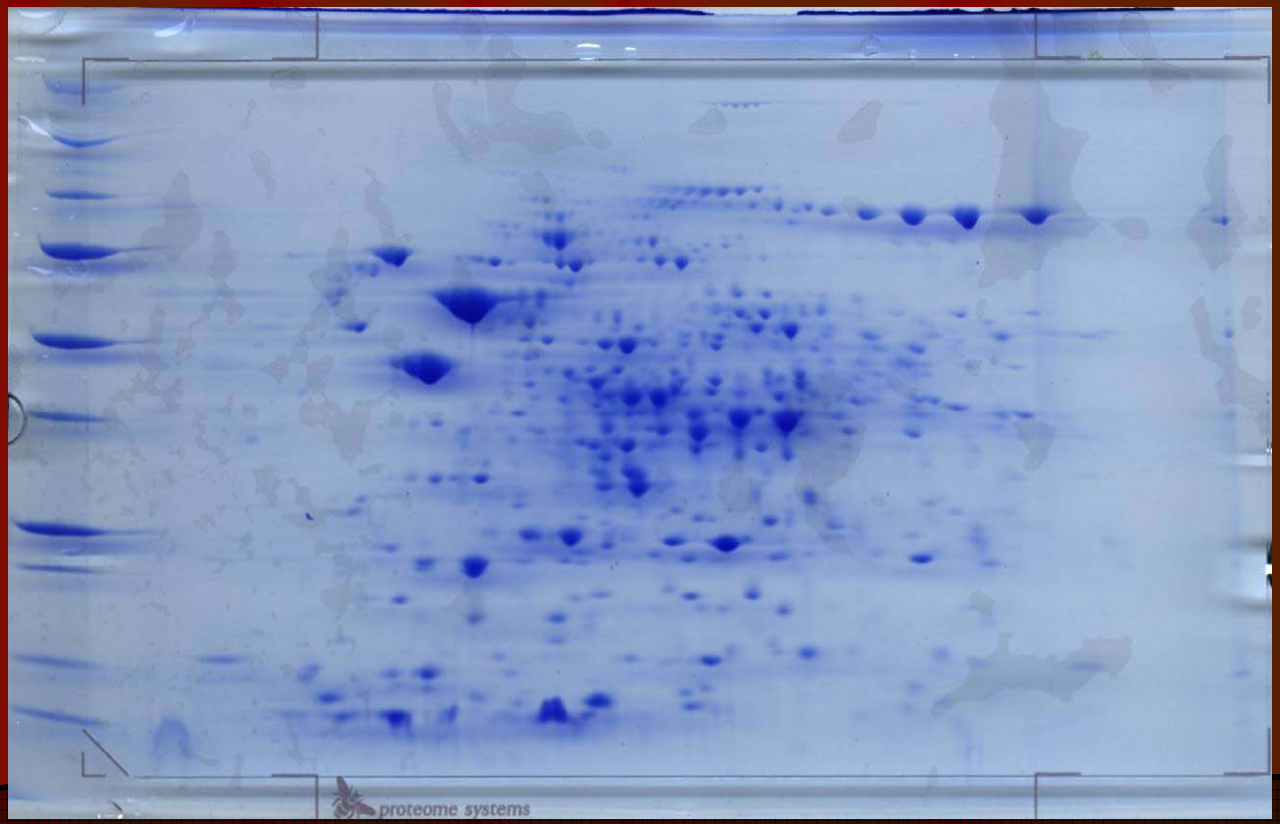
1st Dimension

Isolelectric Focusing

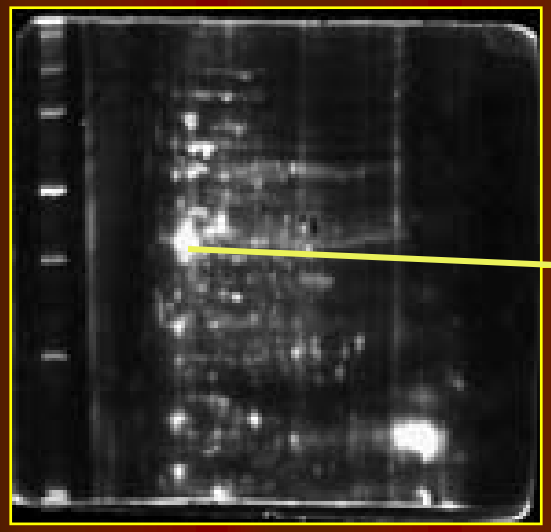
pH 4-7

SDS-PAGE

2nd Dimension



Method of Protein Identification

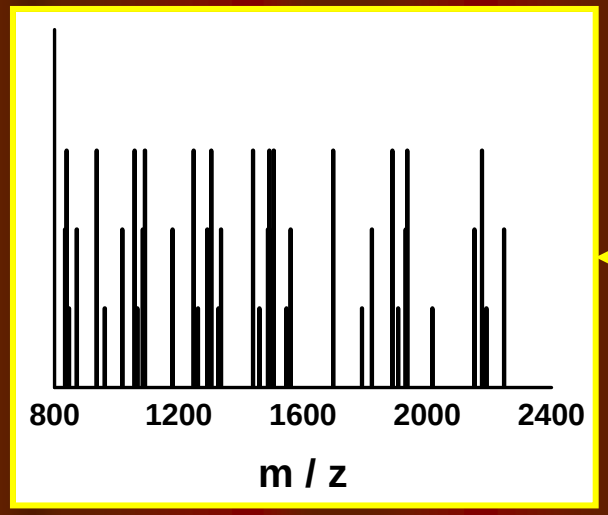


1 - Spot excision

2 - Tryptic digest

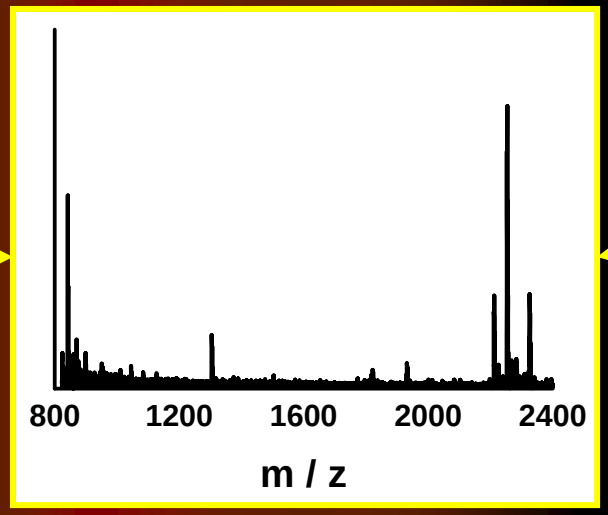
3 - Sample cleanup

4 - Mass spectrometry



theoretical

Mascot



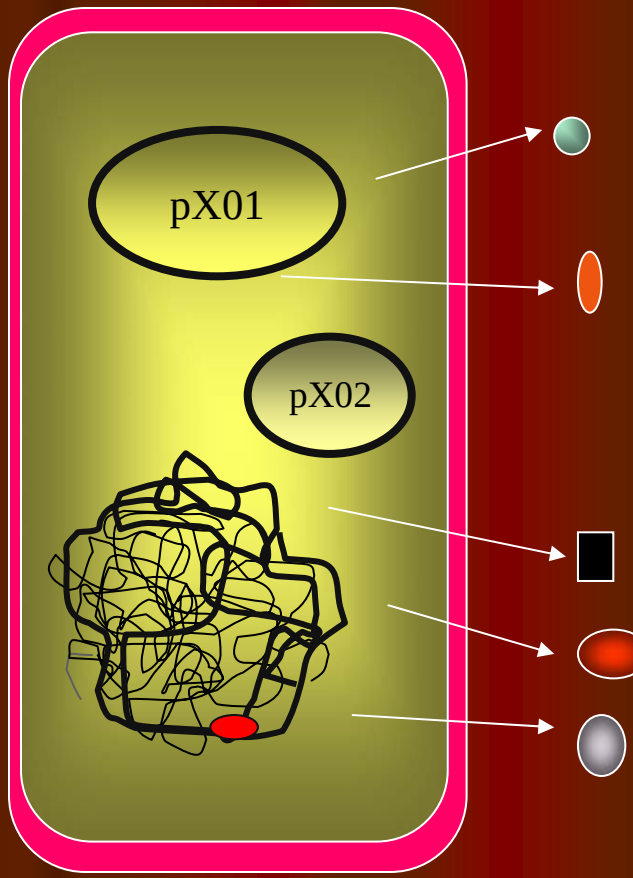
experimental

Protein ID

OBJECTIVE: Target the secretome, membrane, and exosporial proteins of *Bacillus anthracis* to create a shortlist of protein candidates to use in vaccine development.

- These subproteomes contain proteins that help the pathogen invade host tissues.
- They also contain the first factors that confront the host cell.
- Contain many immunogenic proteins.
- Ideal vaccine targets.

Bacillus anthracis Secretome



The secretome corresponds to the proteins exported into the external environment by an organism.

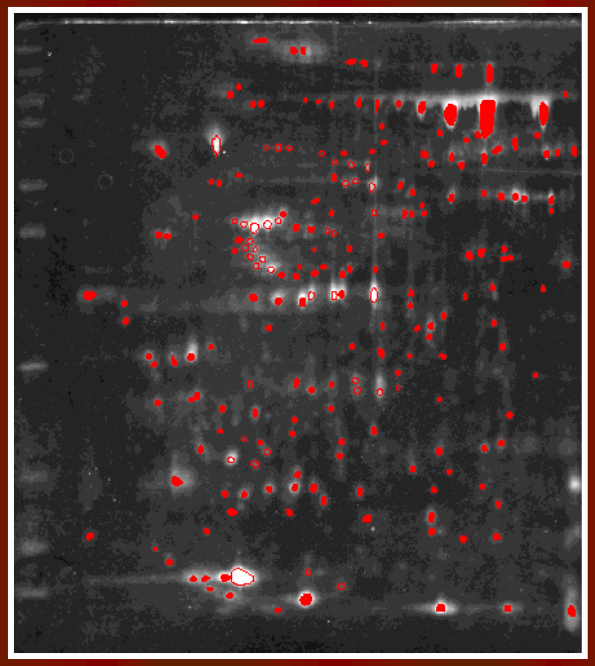
Secreted proteins are often part of the pathogen's early offensive strategy and can be considered remote control virulence factors.

They modify the host cells environment.

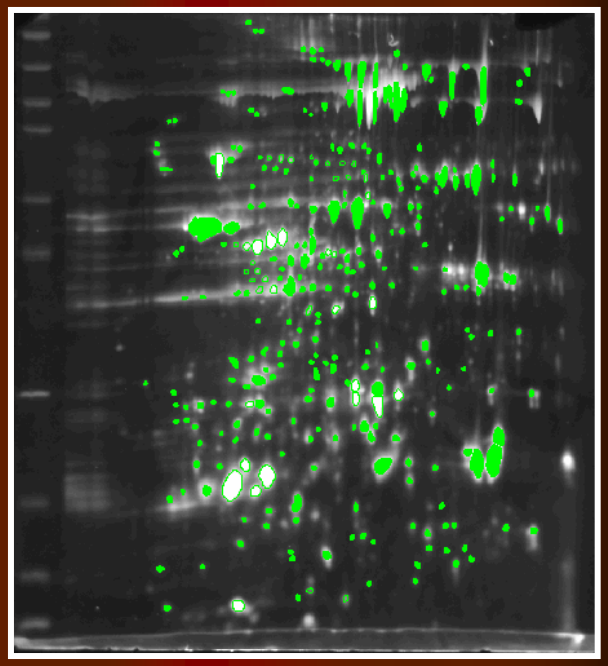
Thus, they are play a major role in the initial host cell/pathogen interactions.

Since they are natural candidates for MHC presentation, they are ideal vaccine targets.

Overview of *Bacillus anthracis* RA3 secretomes (pH 4-7)



Non-induced (254 spots)



Induced (322 spots)

Induction conditions use the R medium which is a minimum synthetic medium developed by Ristroph in 1983 for the production and purification of *B. anthracis* toxins. The culture is grown in a 5% CO2 atmosphere. The conditions simulate those of the host environment.

Vaccine candidate selection is facilitated by identification of immunogenic proteins using immunoblotting of 2D gels and immunoabsorbant columns followed by LC-MS/MS.

Immunoproteomics

Identification and Selection of Immunogenic Proteins | *Clone ORF into Bacterial Ghost vector* | *Bacterial Ghost Production* | *QC of Bacterial Ghosts* | *Animal Studies*

MALDI-TOF

Total Protein

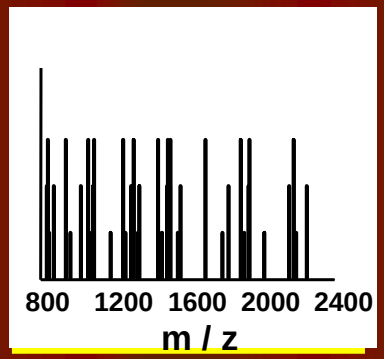
Immunogenic Proteins

1 - Spot excision of immunogenic proteins

2 - Tryptic digest

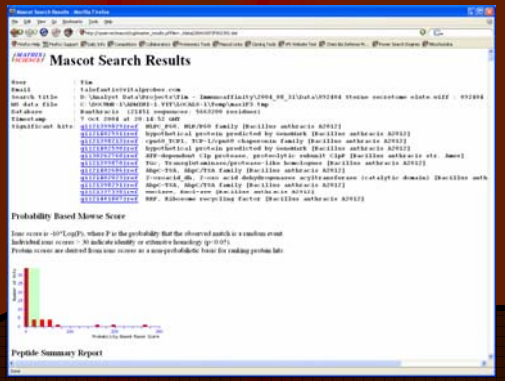
3 - Sample cleanup

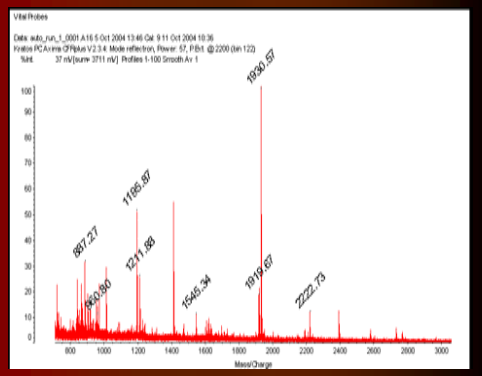
4 - Mass spectrometry



theoretical

Mascot



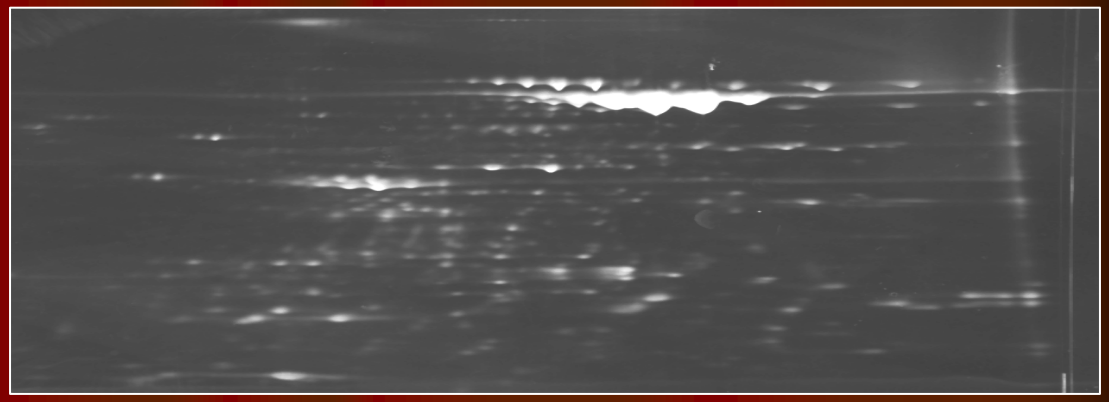


experimental

Protein ID

B. anthracis SECRETOME (CO_2 induced) RA3R (pXO1+pXO2-)

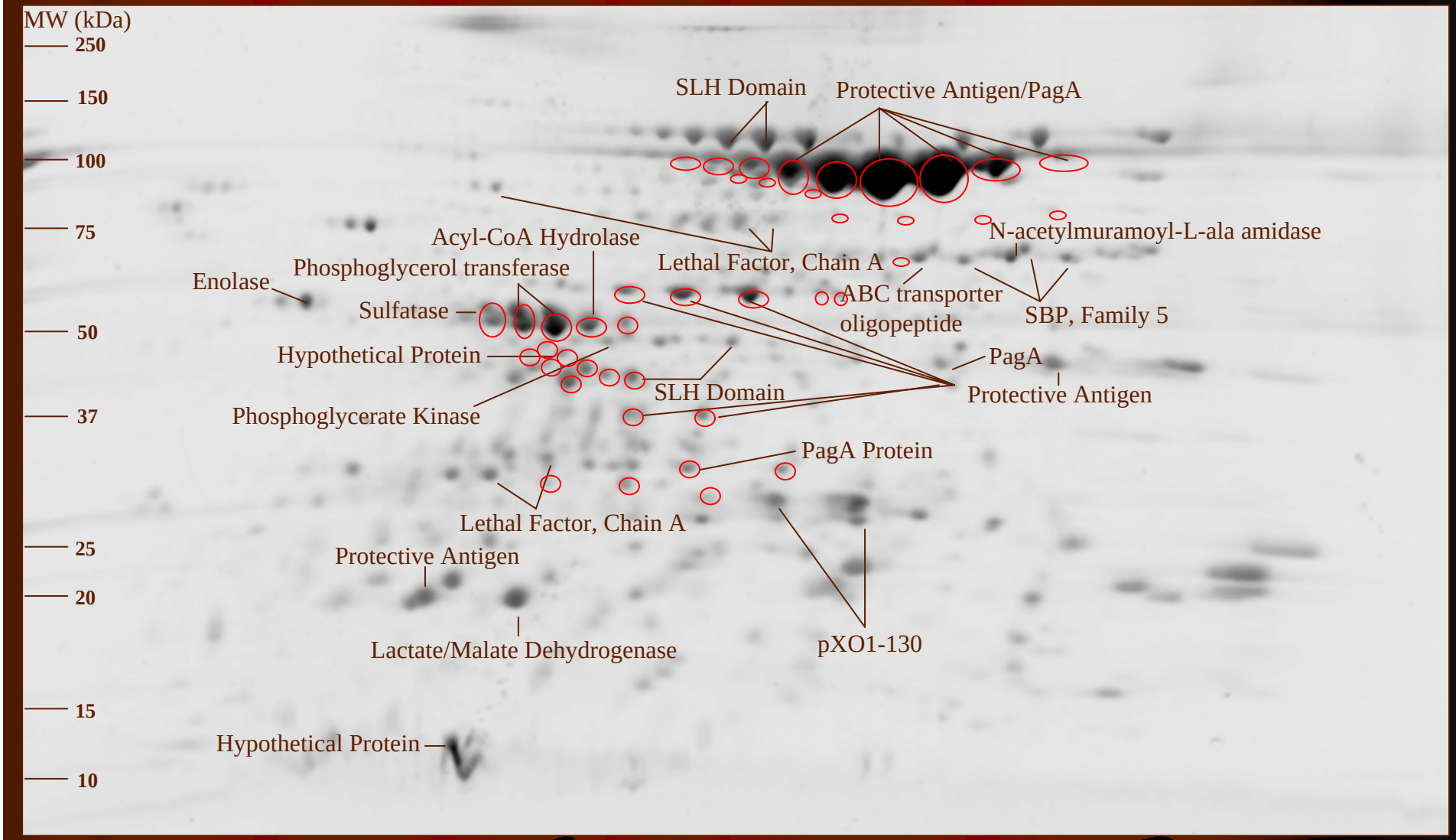
Sypro Ruby Stained gel



Immunoblot Human Ab

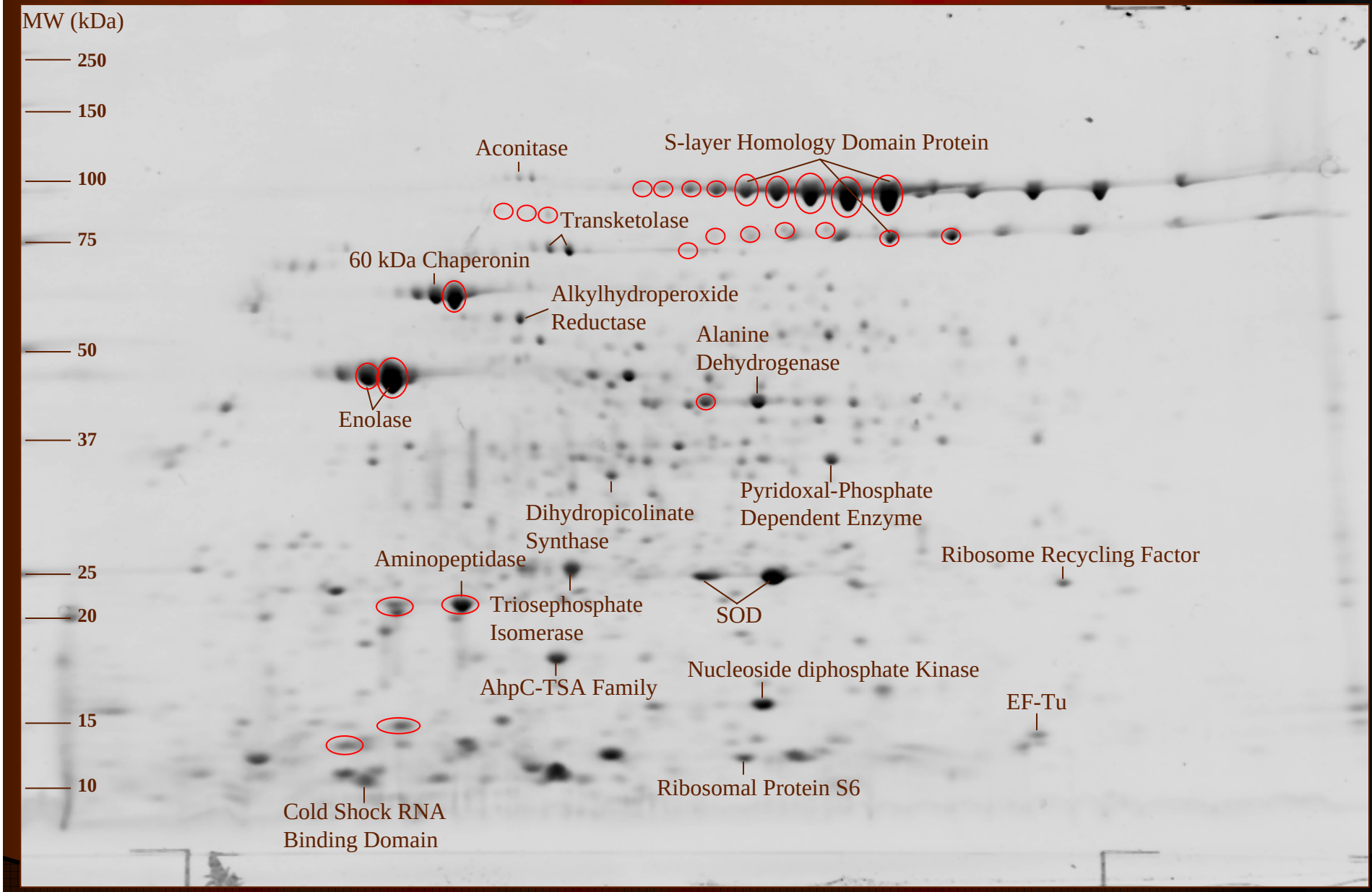
Immunoblot anti glcNAc

Secretome Proteins of *B. anthracis* (pXO1^{+}), pH 4 to 7 (negative image)

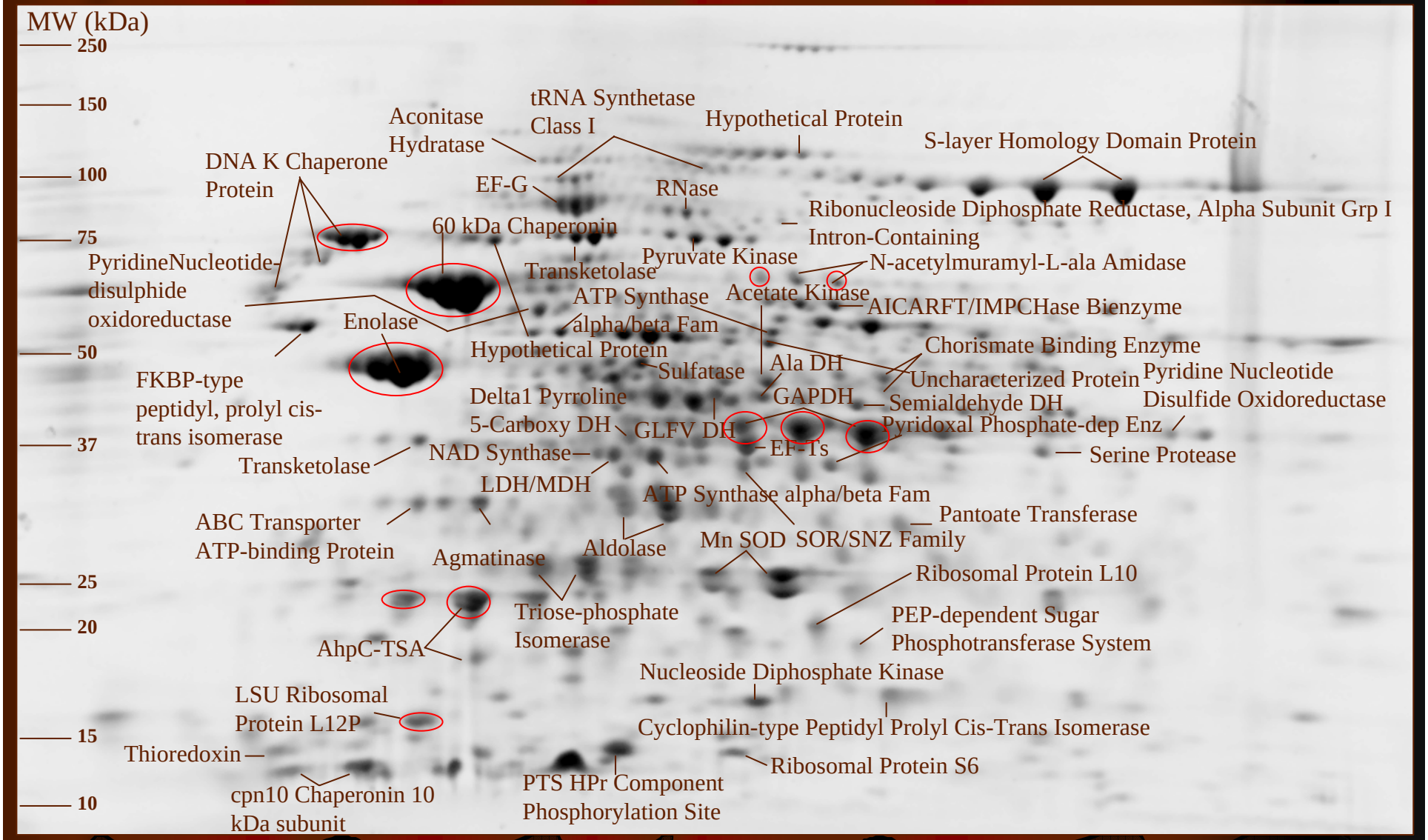


Spots encircled in red represent immunogenic proteins as determined by Western blots.

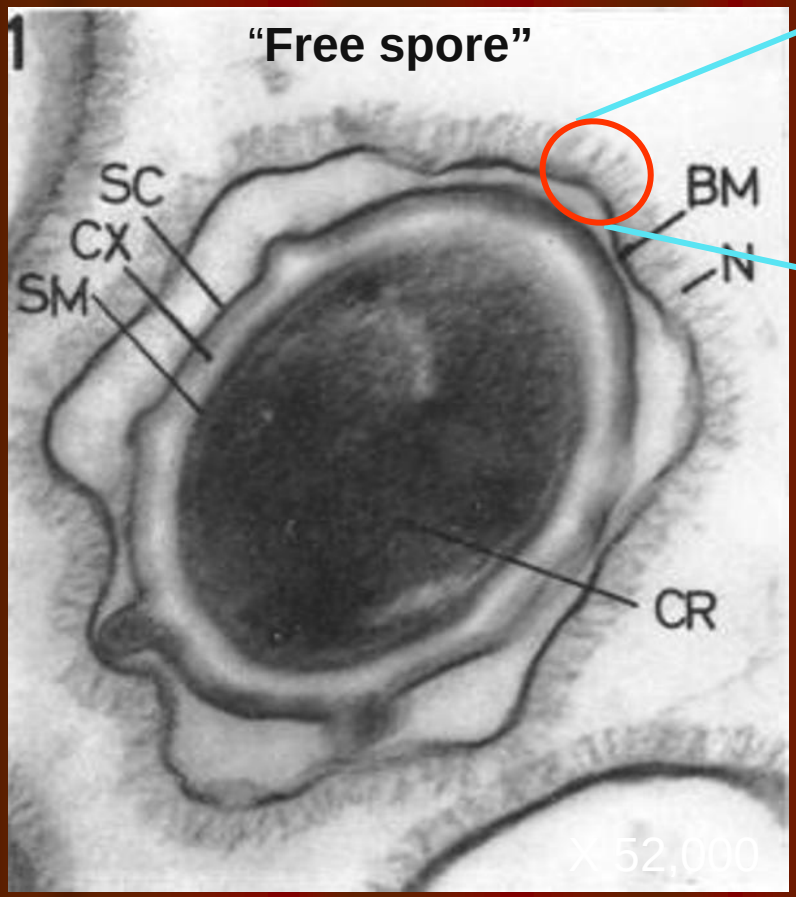
Secretome Proteins of *B. anthracis* (pXO2^{+}), pH 4 to 7 (negative image)



Secretome Proteins of *B. anthracis* (no plasmids), pH 4 to 7



Identification of *Bacillus anthracis* Exosporial Proteins



~ 65% protein

~ 20% lipid

~ 15% carbohydrate

SM: spore membrane

CX: Cortex CR: Core

SC: Spore coat

BM: Basal membrane (crystalline structure)

N: Nap (Hair-like structure)

Exosporial and membrane proteins promote adherence to host cell surfaces.

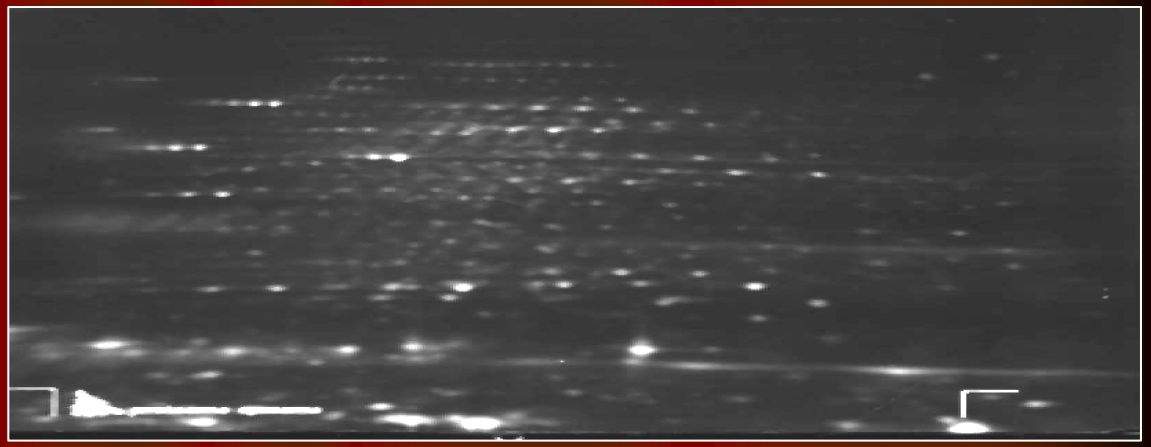
Membrane proteins transport solutes and nutrients, export proteins and macromolecules, allow cell-cell signaling, and sense changes in the environment.

Proteins on the outside of the spore or cell membrane often elicit an immune response.

CAUTION. Immunoreactivity does not always equate with immunoprotection.

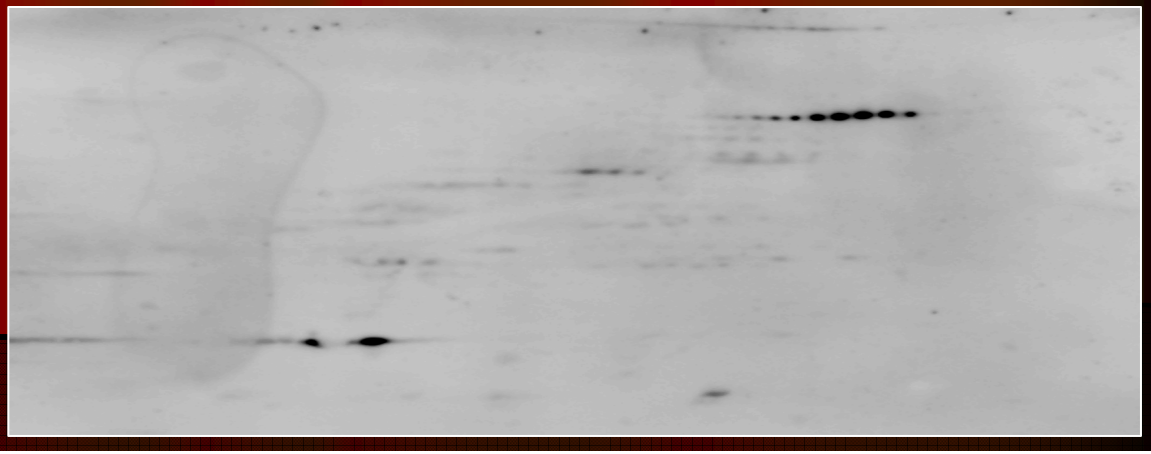
B. anthracis EXOSPORIUM – RA3R (pXO1^{+}, pXO2^{-})

Sypro Ruby Stained gel

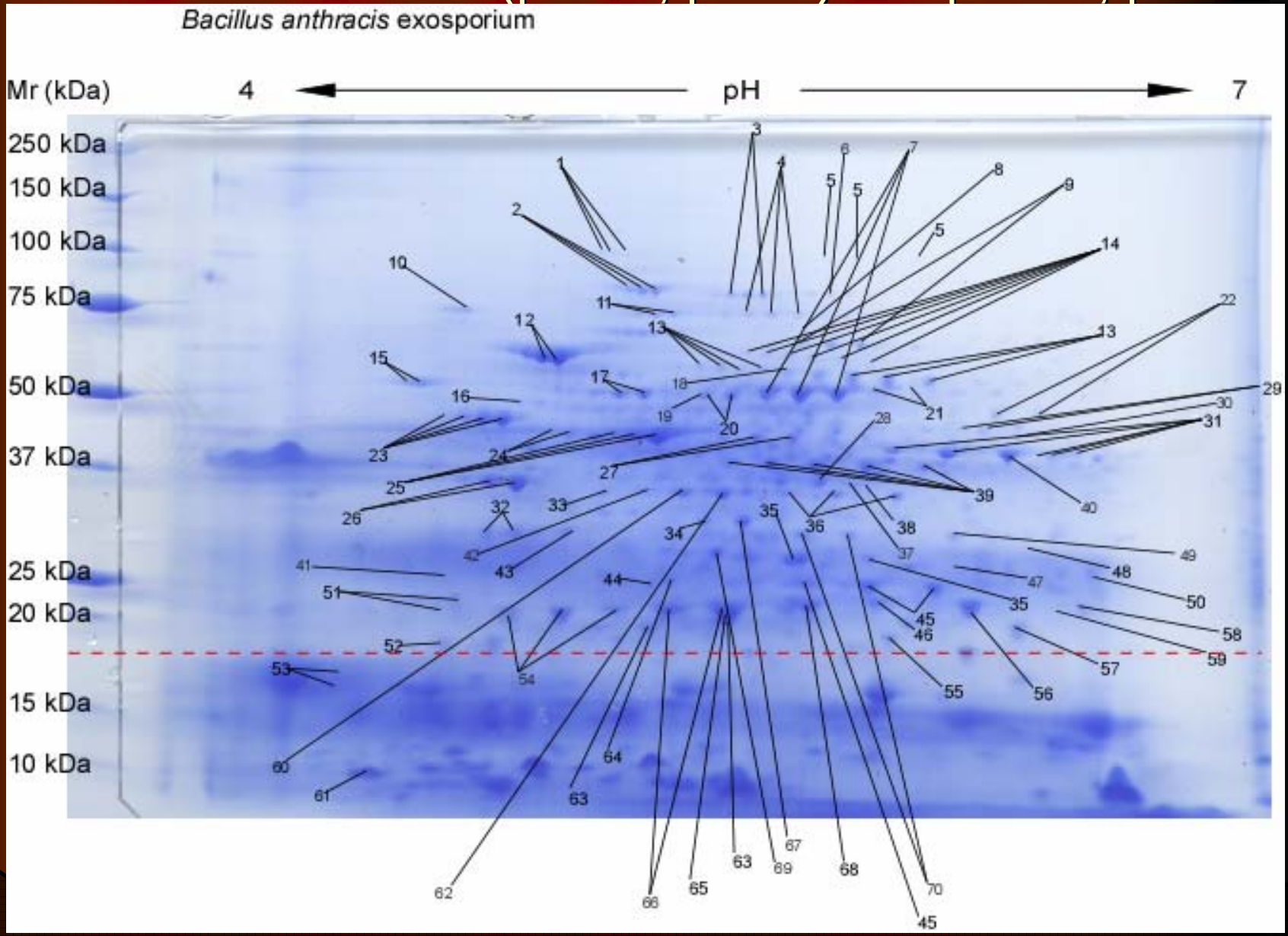


Immunoblot Human Ab

Immunoblot anti glcNAc



Proteome of *Bacillus anthracis* RA3R (pXO1^{+}, pXO2^{-}) exosporium, pH 4-7



Proteome of *Bacillus anthracis* RA3R (pXO1+, pXO2-) exosporium, pH 4-7

spot number	accession no.	theoretical mass	score	protein identification
1	gi|21401538|ref	98977	88	aconitase, Aconitase family (aconitate hydratase) [Bacillus anthracis A2012]
2	gi|21398069|ref	22332	69	GTP_EFTU, Elongation factor Tu GTP binding domain [Bacillus anthracis A2012]
3	gi|21401790|ref	78160	65	RNase_PH, 3' exoribonuclease family [Bacillus anthracis A2012]
4	gi|21402641|ref	62199	104	PK, Pyruvate kinase, barrel domain [Bacillus anthracis A2012]
5	gi|21398845|ref	91307	53	SLH, S-layer homology domain [Bacillus anthracis A2012]
6	gi|21402055|ref	87161	92	Methionine_synt, Methionine synthase, vitamin-B12 independent [Bacillus anthracis A2012]
7	gi|49186878|ref	49423	153	pyruvate dehydrogenase complex E3 component, dihydrolipoamide dehydrogenase [Bacillus anthracis str
8	gi|21399117|ref	70321	60	Peptidase_M3, Peptidase family M3 [Bacillus anthracis A2012]
9	gi|21397814|ref	59716	71	GATase, Glutamine amidotransferase class-I [Bacillus anthracis A2012]
10	gi|21402361|ref	65727	167	HSP70, Hsp70 protein [Bacillus anthracis A2012]
11	gi|21401599|ref	69969	70	transketolase, Transketolase, thiamine diphosphate binding domain [Bacillus anthracis A2012]
12	gi|21398213|ref	32749	92	cpn60_TCP1, TCP-1/cpn60 chaperonin family [Bacillus anthracis A2012]
13	gi|21398255|ref	56190	126	aldedh, Aldehyde dehydrogenase family [Bacillus anthracis A2012]
14	gi|21398556|ref	60967	152	Peptidase_M4_C, Thermolysin metallopeptidase, alpha-helical domain [Bacillus anthracis A2012]
15	gi|21402519|ref	47185	97	FKBP, FKBP-type peptidyl-prolyl cis-trans isomerase [Bacillus anthracis A2012]
16	gi|21402140|ref	46196	54	Glycos_transf_3, Glycosyl transferase family, a/b domain [Bacillus anthracis A2012]
17	gi|21397777	51162	172	ATP-synt_ab, ATP synthase alpha/beta family, nucleotide-binding domain [Bacillus anthracis A2012]
18	gi|21402023|ref	44874	74	2-oxoacid_dh, 2-oxo acid dehydrogenases acyltransferase (catalytic domain) [Bacillus anthracis A201
19	gi|21397379	50313	56	PGI, phosphoglucose isomerase
20	gi|21398266|ref	52262	120	Amidase, Amidase [Bacillus anthracis A2012]
21	gi|21401476|ref	53709	169	aldedh, Aldehyde dehydrogenase family [Bacillus anthracis A2012]
22	gi|49188304|ref	47394	83	adenylosuccinate synthetase [Bacillus anthracis str. Sterne]
23	gi|21397598	46389	188	enolase, Enol-ase [Bacillus anthracis A2012]
24	gi|21398094	34914	68	RNA_pol_A_bac, Bacterial RNA polymerase, alpha chain, N terminal domain [Bacillus anthracis A2012]
25	gi|30018378	42912	168	Protein Translation Elongation Factor Tu (EF-TU) [Bacillus cereus ATCC 14579]
26	gi|21402024|ref	35207	150	transket_pyr, Transketolase, pyridine binding domain [Bacillus anthracis A2012]
27	gi|21402217|ref	39826	80	GLFV_dehydrog, E/Leucine/Phenylalanine/Valine dehydrogenase [Bacillus anthracis A2012]
28	gi|49186676|ref	32415	57	translation elongation factor Ts [Bacillus anthracis str. Sterne]
29	gi|21398197	43635	119	Ala_racemase, Alanine racemase [Bacillus anthracis A2012]
30	gi|30265339|ref	45083	60	serine hydroxymethyltransferase [Bacillus anthracis str. Ames]
31	gi|21400841|ref	40001	58	DAHP_synth_1, DAHP synthetase I family [Bacillus anthracis A2012]
32	gi|21397465|ref	29040	60	ABC_tran, ABC transporter [Bacillus anthracis A2012]
33	gi|21402213|ref	35768	65	transket_pyr, Transketolase, pyridine binding domain [Bacillus anthracis A2012]
34	gi|21397811|ref	30654	95	F_bP_aldolase, Fructose-bisphosphate aldolase class-II [Bacillus anthracis A2012]
35	gi|21401303|ref	23993	79	Transaldolase, Transaldolase [Bacillus anthracis A2012]
36	gi|21398032|ref	32898	151	PALP, Pyridoxal-phosphate dependent enzyme [Bacillus anthracis A2012]
37	gi|21399802|ref	34766	81	ldh, lactate/malate dehydrogenase, NAD binding domain [Bacillus anthracis A2012]
38	gi|21401818|ref	31192	63	CoA_binding, CoA binding domain [Bacillus anthracis A2012]
39	gi|21397602|ref	35803	59	gpdh_C, Glyceraldehyde 3-phosphate dehydrogenase, C-terminal domain [Bacillus anthracis A2012]

Protein spots identified at 

LC-MS/MS Identification of Vaccine Candidate Proteins

Identification and Selection of Immunogenic Proteins

Clone ORF into Bacterial Ghost vector

Bacterial Ghost Production

QC of Bacterial Ghosts

Animal Studies

LC-MS/MS

Sera from B.a. infected human

Apply Protein Sample

Elute Sample

Binding Proteins

Non-binding Proteins

General Immunogenic Protein

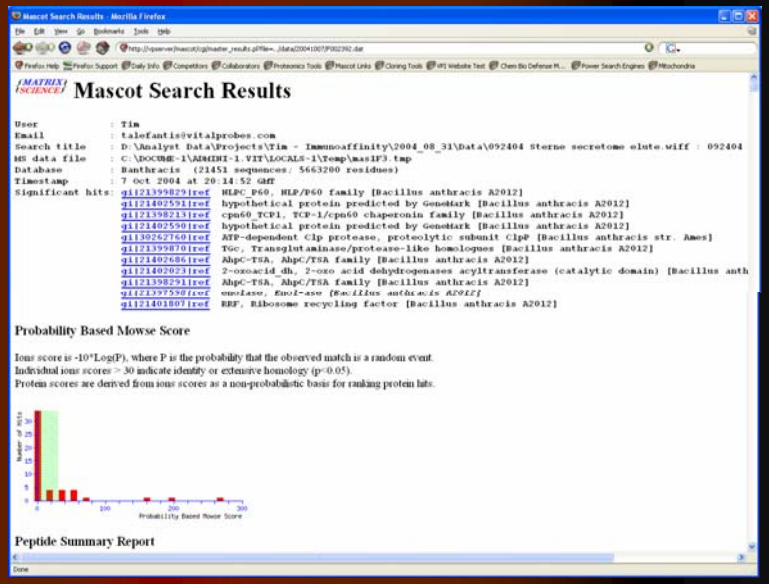
B.a. Specific Immunogenic Proteins

NORMAL Human Sera

Binding Proteins

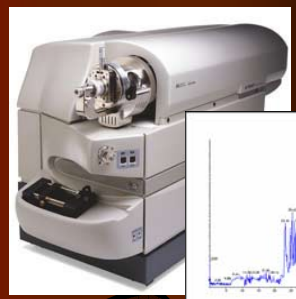
Non-binding Proteins

General Immunogenic Protein

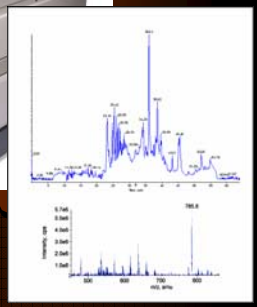


Protein ID

Mass Spec



QTRAP LC-MS/MS



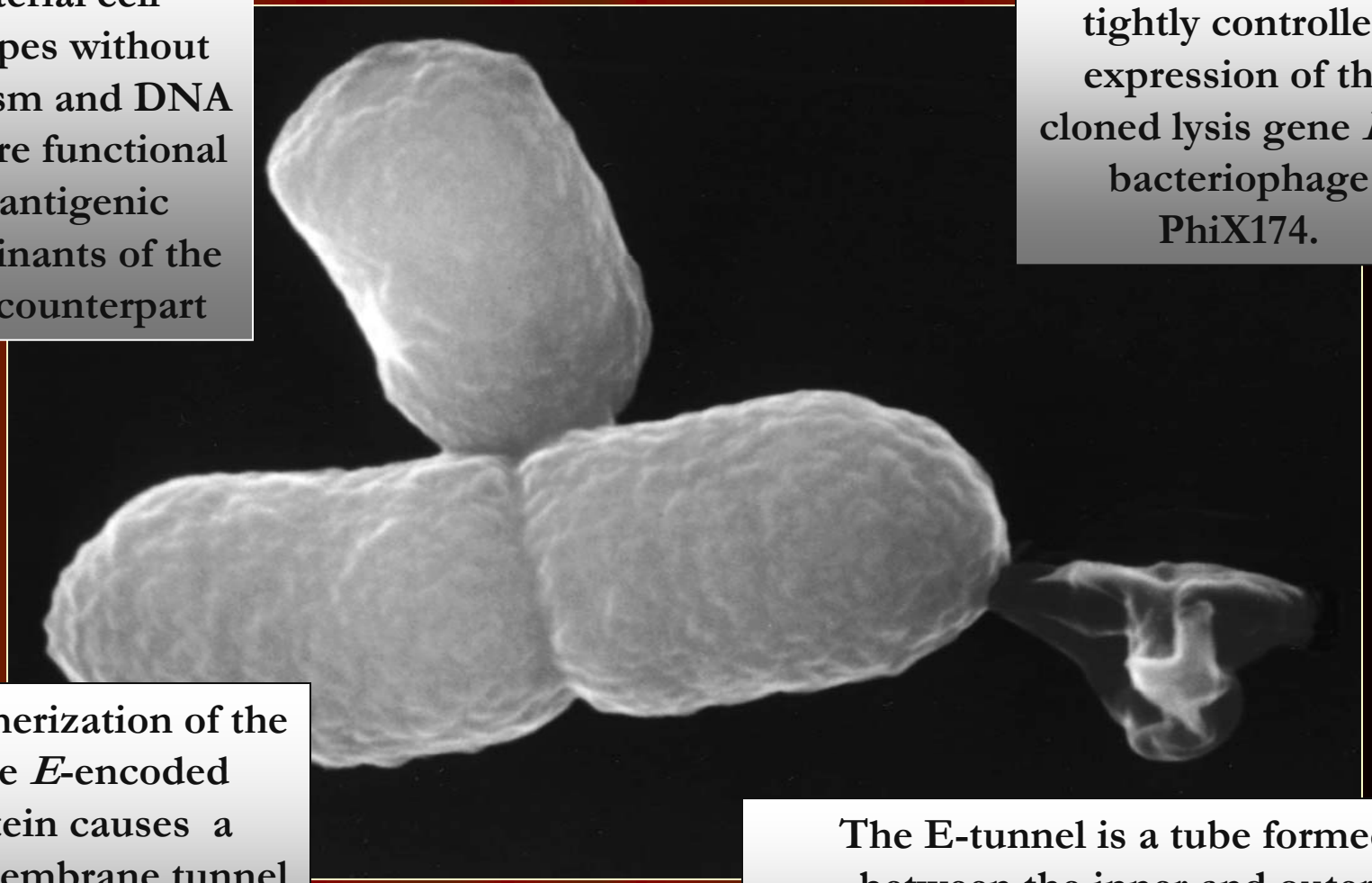
Open Reading Frames Identified in *Bacillus anthracis*

- 64 ORF's identified in exosporium
- 86 ORF's identified in secretome
- a total of 35 of these ORF's are immunoreactive

Bacterial Ghosts

Ghosts are empty bacterial cell envelopes without cytoplasm and DNA but share functional and antigenic determinants of the living counterpart

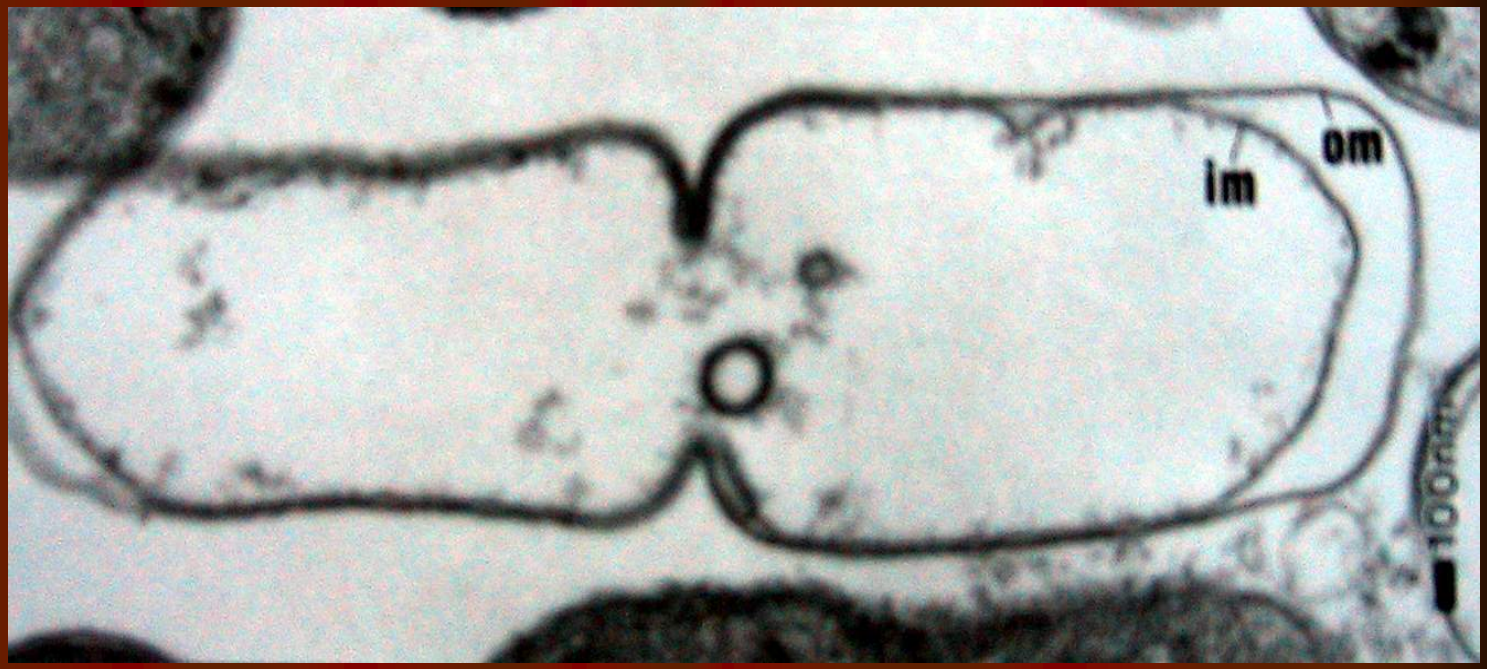
Generated by the tightly controlled expression of the cloned lysis gene *E* of bacteriophage PhiX174.



Oligomerization of the gene *E*-encoded protein causes a transmembrane tunnel in the bacterial cell wall of gram-negative bacteria.

The E-tunnel is a tube formed between the inner and outer membranes. The E-tunnel causes the cytoplasm of the cell to escape, forming the ghost.

Transmission Electron Micrograph of Bacterial Ghost with E-tunnel



Advantages of Bacterial Ghosts

Nonliving alternatives
to
Chemical
Irradiated
Heat
Inactivated bacteria

Production process
Does not denature
The bacterial ghost
envelope

Bacterial Ghosts
Can be produced
In large quantities
By fermentation

Stable as freeze-dried
Material for
long periods
of time and
Do not require
cold storage

Current ongoing studies demonstrate that bacterial Ghosts are stable at ambient temperature for at least 5 years (to date)

Advantages of Bacterial Ghosts

Bacterial Ghosts have strong adjuvant activity

Bacterial Ghosts are easily Self Administered

Bacterial ghosts can Be delivered by
Oral
Respiratory
Conjunctival
Subcutaneous
Intramuscular
Routes of administration

Bacterial Ghost cocktails can easily be used to create multi-agent vaccines

Vaccine Development Workflow: Proteomics to Bacterial Ghosts

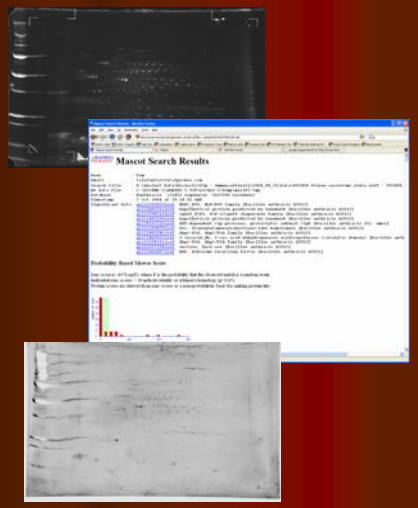
Identification of Immunogenic Proteins

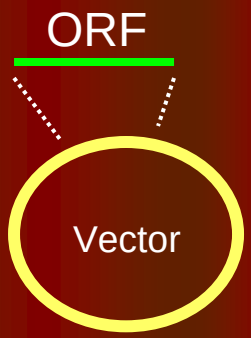
Clone ORF into Bacterial Ghost vector

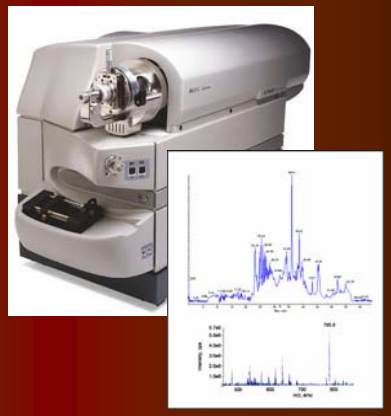
Bacterial Ghost Production

QC of Bacterial Ghosts

Animal Studies







Vaccine Candidate Selection Criteria

Identification and Selection of Immunogenic Proteins | *Clone ORF into Bacterial Ghost vector* | *Bacterial Ghost Production* | *QC of Bacterial Ghosts* | *Animal Studies*

Species Immunogenicity

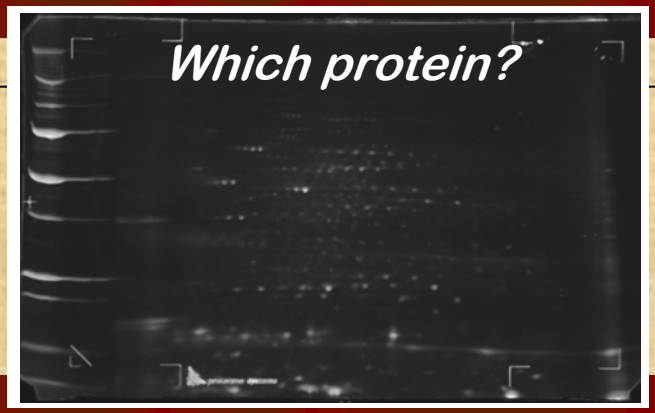
Human or Rabbit

H + R = 3 points
H only = 2 points
R only = 1 point

Location

Exosporia, Secretome, Membrane

3 sites = 3 points
2 sites = 2 points
1 site = 1 point



Degree of Immunogenicity

High = 3 points
Med = 2 points
Low = 1 point

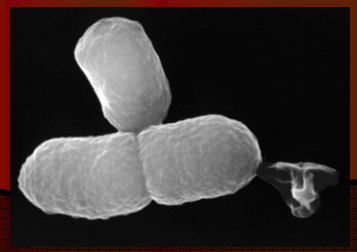
Degree of Immunogenicity =

$$\frac{\text{Western Blot Spot Volume}}{\text{SYPRO Spot Volume}}$$

Use in other vaccines

Protein used previously?

Protective = 3 points
Good Candidate = 2 points
No Mention = 1 point



Homology to target organism

Human, Bovine, etc

None = 3 points
Moderate = 2 points
High = 1 point

VITAL PROBES

Vaccine Candidate Selection Criteria

Identification and Selection of Immunogenic Proteins | *Clone ORF into Bacterial Ghost vector* | *Bacterial Ghost Production* | *QC of Bacterial Ghosts* | *Animal Studies*

Compile data to produce single score per protein…

	Location		Species Immunogenicity		Degree of Immunogenicity		Similar to protein used in other vaccine development studies		Homology to Human Proteins		SCORE
									bits		
Alanine Racemase	E	1	H	2	Med	2	Good Candidate	2	0	2	18.1
Dehydrogenase E1 Component	E	1	H	2			Good Candidate	2	165	0	8.9
Protective Antigen	S	1	H	2	High	3	Protective	3	0	2	20.9
LSU Ribosomal Protein L12P	E, S	1	H	2	Med	2	Protective	3	0	2	19.3
AhpC-TSA	E, S, M	3	H, R, Gu	3	High	3	Good Candidate	2	150	0	20.7
Oligopeptide ABC transporter (OppA)	M	1	R	1			No Mention	1	0	2	11.7

Location

Exosporia, Secretome, Membrane

3 sites = 3 points
2 sites = 2 points
1 site = 1 point

Species Immunogenicity

Human, Rabbit, Goat

3 species = 3 points
2 species = 2 points
1 specie = 1 point

Degree of Immunogenicity

High = 3 points
Med = 2 points
Low = 1 point

Use in other vaccines

Protein used previously?

Protective = 3 points
Good = 2 points
Candidate
No Mention = 1 point

Homology to target organism

Human, Bovine, etc

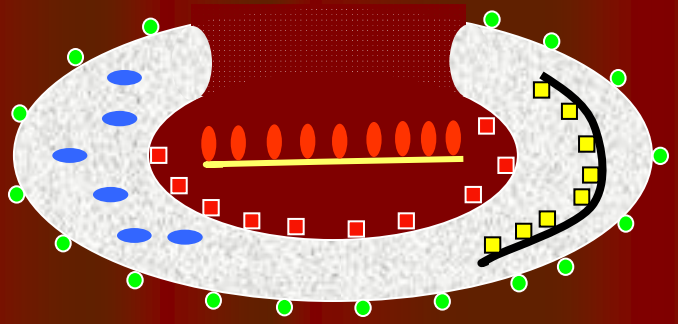
None = 3 points
Moderate = 2 points
High = 1 point

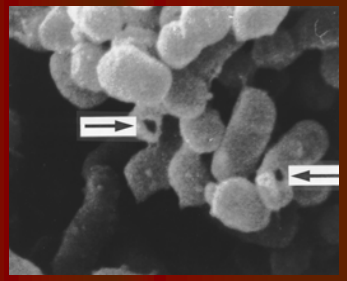
VITAL PROBES

Placement of Recombinant Protein on Bacterial Ghosts

Identification and Selection of Immunogenic Proteins | *Clone ORF into Bacterial Ghost vector* | *Bacterial Ghost Production* | *QC of Bacterial Ghosts* | *Animal Studies*

Locations on bacterial ghosts where expressed proteins can be directed





- Outer Membrane
- Periplasmic Space
- Inner Membrane
- Cytoplasm – S-Layer
- Periplasmic Space – S-Layer

Production of Bacterial Ghosts

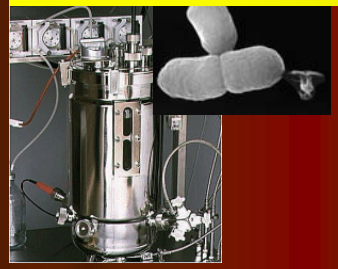
Identification and Selection of Immunogenic Proteins | *Clone ORF into Bacterial Ghost vector* | ***Bacterial Ghost Production*** | *QC of Bacterial Ghosts* | *Animal Studies*

Preparation

Transformation of host cell (*E.* coli K12 (NM522)) with

- Plasmid containing gene of interest
- Plasmid containing genes for
  - E-mediated lysis
  - Digestion of DNA (Staph nuclease)

Production



Culture at 28C to produce required level of bacteria

Induce recombinant protein production with IPTG

Induce E-lysis (and Staph nuclease)

- Thermal induction (39-42C)

Harvest ghosts

Quality Control

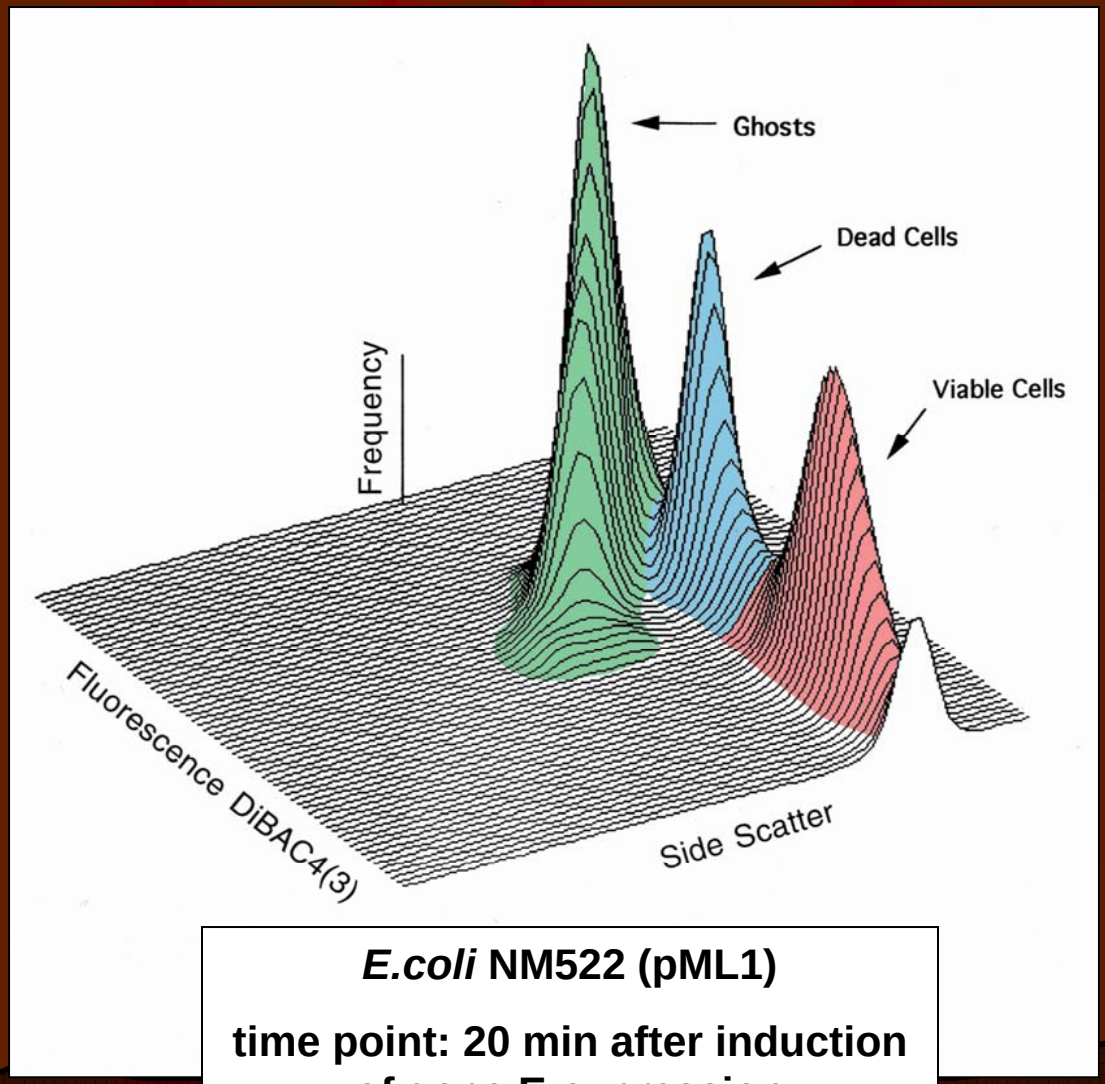
Viability Analysis

- Colony forming assay
- FACS analysis

Recombinant Protein Analysis

- 2D Gel analysis (western blot analysis)
- LC-MS/MS analysis (quantification)

Online monitoring of bacterial ghost production by flow cytometry



***E.coli* NM522 (pML1)**

time point: 20 min after induction of gene E-expression

Future projects

Vaccines for other BWA.

Fill BG with antimicrobial agents.

Use BG as carriers of DNA vaccines.